Structure and activity studies of tyrosinases and related proteins
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5. Summary and Outlook

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1. Summary
In this thesis the structure and activity are investigated of tyrosinases and related proteins, including mushroom tyrosinase-associated lectin-like protein (MtaL), human tyrosinase (TYR) and human tyrosinase related protein 1 (TYRP1).

1.1 Tyrosinase-associated lectin-like protein (MtaL)
In Chapter 2 we report the 1.35 Å resolution crystal structure of recombinant MtaL (Lai et al., 2016a). MtaL has been previously characterized as a lectin-like protein that binds to mature Agaricus bisporus tyrosinase in vivo, but its exact physiological function is unknown (Ismaya et al., 2011). Comparison of the structure of recombinant MtaL with that of the truncated and cleaved MtaL present in the complex with tyrosinase directly isolated from mushroom shows that the general β-trefoil fold is maintained (Ismaya et al., 2011). However, differences are detected in the loop regions, particularly in the β2-β3 loop (chapter 2, Fig. 4), which is intact and not cleaved in the recombinant MtaL. Furthermore, the N-terminal tail is rotated inwards, covering the tyrosinase-binding interface. Thus, MtaL must undergo conformational changes in order to bind mature mushroom tyrosinase. Very interestingly, the β-trefoil fold has been identified to be essential in other lectin-like proteins for interaction with carbohydrates (Arndt et al., 2005; Pohleven et al., 2012). Comparison of the structures of MtaL and a ricin-B-like lectin with a bound disaccharide shows that MtaL may have a similar carbohydrate-binding site that might be involved in glycoreceptor activity (Lai et al., 2016a).

1.2 Human tyrosinase (TYR)
In Chapter 3 we report the large-scale recombinant expression and purification of human tyrosinase (TYR) suitable for structural studies (Lai et al., 2016b). TYR is a type 3 copper-containing glycoprotein that initiates the first two reactions in the melanin biosynthesis pathway (Sánchez-Ferrer et al., 1995; Solomon et al., 1996). Mutations in its encoding gene cause Oculocutaneous Albinism type I (OCA1), the most severe form of albinism, which is a group of autosomal recessive disorders characterized by reduced or absent production of melanin in skin, hair and eyes (King et al., 2003; Oetting et al., 2003). Despite extensive structural and characterization studies of its homologues in lower eukaryotic organisms, the catalytic mechanism of TYR and the molecular basis of OCA1 are largely unknown. In this chapter, we carried out a large-scale recombinant expression of TYR that has enabled us to obtain high yields of pure and active protein, suitable for crystallization trials and for screening for skin whitening agents, which is highly demanded in the cosmetic industry. Addition of an N-terminal honeybee melittin signal peptide for secretion of the produced protein into the (protein-free) medium, as well as incorporation of a cleavable His-tag at the C-terminus, were crucial for increasing the yield of pure protein (Tessier et al., 1991). We have successfully crystallized two TYR variants, in both glycosylated and deglycosylated forms, showing preliminary X-ray
diffraction patterns extending to 3.5 Å resolution. Hence, we established an expression and purification protocol suitable for the crystal structure determination of TYR, which will give unique atomic insight into the nature and conformation of the residues that shape the substrate-binding pocket. Such insight may ultimately lead to efficient inhibitor design.

Unfortunately, our attempts to solve the crystal structure of TYR using the 3.5 Å diffraction data set were not successful. The crystals seem to be tetartohedrally twinned, and molecular replacement only yielded a partial solution with 10 protomers in the asymmetric unit of space group $P_3_2$ (data not shown). Model building and refinement was challenging due to the low resolution and the twinning. Nevertheless, for other proteins, tetartohedrally twinned crystals have given good structure solutions in some cases when the diffraction limit was increased (Roversi et al., 2012). Thus, future perspectives are set to increase the diffraction limit of the crystals by various strategies that have been shown to be successful for several other proteins, such as crystal optimization including seeding (Bergfors, 2003; Stura and Wilson, 1990) and additive screening (Chayen and Saridakis, 2008), and crystal post-manipulation including dehydration (Bowler et al., 2006; Sanchez-Weatherby et al., 2009) and annealing (Hanson and Bunick, 2007).

1.3 Human tyrosinase-related protein 1 (TYRP1)

In chapter 4 we report the crystal structures of human tyrosinase related protein 1 (TYRP1) in its unliganded state and in various ligand-bound states. TYRP1 is hitherto considered to also be a type 3 copper-containing glycoprotein that, together with tyrosinase and tyrosinase related protein 2 (TYRP2), belongs to the so-called melanogenic proteins, which are involved in the mammalian melanin synthesis pathway (Hearing and Tsukamoto, 1991). TYRP1 has been considered to be a DHICA (5,6-dihydroxyindole-2-carboxylic acid) oxidase (Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994a, 1994b), although other functions have also been proposed, such as catalase (Halaban and Moellmann, 1990), tyrosine hydroxylase (Zhao et al., 1994) and L-DOPA oxidase (Jimenez-Cervantes et al., 1993).

The crystal structure of TYRP1 is the first mammalian tyrosinase-like structure ever elucidated. The intra-melanosomal domain of human TYRP1 reveals a compact globular fold built up of two tightly interacting subdomains, an N-terminal Cys-rich subdomain and a tyrosinase-like subdomain. The tyrosinase-like subdomain has the typical tyrosinase fold, with a core of four helices that make up the active site, with contains the binuclear type 3 metal binding site. The Cys-rich subdomain is unique to vertebrate melanogenic proteins and its core structure is formed by two pairs of short antiparallel β-strands from which long loops emerge. The subdomain is stabilized by five disulfide bonds, where the central three follow the $[C_1-C_3, C_2-C_4, C_5-C_6]$ signature pattern of epidermal growth factor (EGF)-like structures (Jackson et al., 1992). It interacts with the tyrosinase-like subdomain via its N-terminus and a long loop emerging from the
EGF-like core. The Cys-rich subdomain is located far from the active site, at the opposite side of the molecule, and thus it is unlikely to affect the catalytic activity of TYRP1. Nevertheless, it has been shown that its disulfide bonds are crucial for folding and maturation of melanogenic proteins (Negroiu et al., 2000). Immature TYRP1 that cannot form the correct disulfide bonds is retained in the endoplasmic reticulum and targeted for degradation (Negroiu et al., 2000). Thus, although the precise function of the Cys-rich subdomain is still elusive, correctly linked disulfide bonds are needed for maturation and obtaining functional TYRP1.

Unexpectedly, when assessing the nature of the two metal ions in the TYRP1 active site using X-ray fluorescence and single-wavelength anomalous diffraction, we discovered that TYRP1 contains two zinc ions in its active site, in contrast to tyrosinase, which contains copper ions. This finding indicates that it is unlikely that TYRP1 is a redox enzyme, as had been assumed for the last three decades. Although the exact catalytic function of TYRP1 still remains unknown, we have been able to confer (low) DHICA oxidase and tyrosinase activity to TYRP1 by replacing zinc with copper ions. However, whether copper incorporation also occurs in vivo is unclear, and needs further investigation.

In view of the similarity of TYRP1 to tyrosinases, we investigated the binding mode of well-established tyrosinase substrates and inhibitors, including L-tyrosine, L-mimosine, kojic acid and tropolone. The four ligands bind in the active site in a similar way as in tyrosinases. Conserved interactions are found, including stacking interactions between the aromatic ring of the ligand and the His381 side chain, and hydrogen bonding between the C-keto group or hydroxyl group of the ligand and the hydroxyl group of Ser394, a residue that is conserved in mammalian tyrosinases, but not in tyrosinases from lower organisms. Furthermore, to investigate how amino acid differences between the active sites of TYRP1 and TYR affect the substrate-binding mode, we solved a structure of TYRP1-3M (TYRP1Y362F-R374S-T391V), a triple mutant that mimics the active site of TYR, with bound L-mimosine. Interestingly, we observed that mimosine binds in a similar conformation as in native TYRP1, despite removal of hydrogen-bonding interactions by the Tyr362Phe, Thr391Val, and Arg374Ser mutations. Two of the three mutated residues, Tyr362 and Arg374, are located at the entrance to the active site, and have been proposed as “substrate-guiding” residues in a plant tyrosinase (Bijelic et al., 2015). However, in TYRP1 such interactions do not appear to be essential since the Tyr362Phe and Arg374Ser mutations do not change the binding mode of mimosine. Further structures of TYRP1-3M with bound tropolone and kojic acid, respectively, show almost identical binding modes as in wild-type TYRP1. Taken together, our findings suggest that the presence/absence of “substrate-guiding residues” at the active site entrance does not affect the binding mode of TYRP1 substrates and inhibitors.

Lastly, the structure of TYRP1 provides a structural rationalization of the effect of disease-related mutations, in particular for oculocutaneous albinism type III
(OCA3) (Sarangarajan and Boissy, 2001), and thus may offer an atomic explanation of the disease mechanism. Such information is useful for efficient genetic counselling and gene therapy, since many reported OCA mutations are based on statistical analysis (Cooper, 1998), without direct proof that they cause the disease.

2. Outlook

2.1 A model of human tyrosinase can give insights into the structural basis of oculocutaneous albinism type I (OCA1)

Our TYRP1 crystal structure is the first structure of a mammalian melanogenic enzyme. In view of the ~40% amino acid sequence identity and ~70% similarity among the three melanogenic proteins, and their very similar domain architecture, the TYRP1 structure can give insights into the functioning of TYR and explain the structural basis of OCA1 related mutations. Thus, using CHAINSAW (Stein, 2008), we have generated a model of TYR covering residues 19 to 455 (corresponding to residues 25-470 in TYRP1) in order to map the OCA1 related mutations (Figure 1). According to the Human Gene Mutation Database, 234 missense/nonsense mutations are associated with OCA1, affecting 169 unique amino acids, of which 162 are part of the current model. Interestingly, out of the 96 residues in the Cys-rich subdomain (residues 19-114), more than one third (i.e. 36 residues) have been implicated in OCA1, which provides further evidence about the relevance of this domain in vertebrate tyrosinases. In particular, 8 out of the 10 disulfide bond-forming cysteines (Cys24, Cys35, Cys36, Cys46, Cys55, Cys89, Cys91 and Cys100) (Figure 1, highlighted in red) are associated with OCA1, except Cys103 and Cys112 (Figure 1, in orange). Other mutations in this subdomain are irregularly distributed, which may indicate the general importance of the domain for the overall folding, and/or maturation or processing of the protein in vivo.

Not surprisingly, all six copper-coordinating histidines are among the severely pathological mutations (Figure 1, in blue sticks). Although only His211 (Figure 1, indicated in red) has been assigned to OCA1A, a subtype of the OCA1 disease in which patients suffer from life-long absence of melanin, our structure suggests that the other five histidines (His180, His202, His363, His367 and His390) would also result in OCA1A (Figure 1, in blue), since those histidines are critical ligands for coordinating the copper cofactors in the active site, and thus, substitution of any of them would yield an inactive enzyme.

Furthermore, a subset of OCA1 related mutations, including R402Q (Berson et al., 2000; Fukai et al., 1995), P406L (Giebel et al., 1991a) and R422Q (Giebel et al., 1991b; Wang et al., 2005), cause the so-called temperature-sensitive oculocutaneous albinism (OCA1-TS). This is related to a temperature-sensitive form of TYR with optimal tyrosinase activity at temperatures lower than 37 °C. As a result, pigment is generally more prominent in the extremities (ears, face and legs), where the temperature is lower than in other parts of the body (King et
In our TYR structure model (Figure 1) Arg402 (displayed as pink sticks and surface; Arg416 in TYRP1) points into the solvent and forms a hydrogen bond with Gln399 (equivalent to Glu413 in TYRP1). Mutation of the Arg into a Gln would likely remove this hydrogen bond since Gln is shorter than Arg and cannot reach Gln399. The removal of one (presumably stabilizing) hydrogen bond in the protein might affect the stability of the protein, causing it to (partially) unfold at higher temperatures, but not at lower temperatures. Similarly, Arg422 (Arg436 in TYRP1) points into the solvent, but has a hydrogen-bonding interaction with the carbonyl oxygen of Phe425 (TYRP1 numbering). There is sufficient space to replace Arg422 by a Trp or Gln. A Gln would also be able to make a hydrogen bond with the carbonyl oxygen atom of Phe425, but not a Trp. Instead, a Trp would have good hydrophobic interactions with Phe425, Leu422, and Leu427. Since a hydrogen bond with a Gln will be weaker than with a positively charged Arg, this may lead to a decreased stability. These two mutation examples show that replacement of Arg402 or Arg422 by another residue generally removes one intra-protein hydrogen bonding interaction, thereby presumably decreasing the stability. The reduced stability can explain the temperature-sensitive behavior of the mutant protein. Yet, since the residues are located on the surface of the protein, no large conformational changes are needed to accommodate them. Finally, Pro406, which corresponds to Ala420 in TYRP1, is located in a loop on the surface of the protein, likely rigidifying the main chain conformation and stabilize the loop. Replacing Pro by a hydrophobic Leu would introduce more flexibility, resulting in protein with lower thermal stability.

Three residues in the vicinity of the active site, including Val377, Ser380 and Glu345 have also been annotated as pathological mutations although the derived subtype of OCA1 has not been reported yet. To investigate their effect on the activity of the protein, we generated three mutants, Val377Ala (Preising et al., 2011), Ser380Pro (Spritz et al., 1997) and Glu345Gly (Opitz et al., 2004) (Figure 1, labelled red sticks). According to the activity assays (Figure 2), Val377Ala still shows some tyrosine hydroxylase and L-DOPA oxidase activities, although they are significantly decreased, suggesting that Val377Ala might cause OCA1B, in which lower amounts of melanin are produced due to the lower activity of the TYR variant. In contrast, both Ser380Pro and Glu345Gly show residual L-DOPA oxidase activity but a completely abolished tyrosine hydroxylase activity. Thus, the two mutations cause the abolishment of the initiating reaction that would block the downstream reactions in the melanin synthesis pathway, resulting in the most severe form of albinism OCA1A, in which no pigment is produced.
Figure 1. OCA1-derived mutations mapped in the model of TYR. The Cys-rich subdomain is shown in green and the tyrosinase-like subdomain in blue. Annotated OCA1-derived mutated disulfide bonds are mapped in red while the additional disulphide bond from the cluster is in orange. The binuclear copper site ions are shown in orange spheres. The metal binding histidines are shown in blue sticks and labelled. The annotated OCA1A-derived His mutation is labelled in red. OCA1-TS mutations are labelled and shown as pink sticks and surface. Experimentally tested mutations (V377, E345 and S380) are show in red sticks. The N and C labels correspond to the N and C-terminus, respectively.

Figure 2. Activity assays with TYR OCA1-related mutants. Spectroscopic assays for (A) tyrosine hydroxylase and (B) L-DOPA oxidase activities, respectively, using recombinant human TYR (residues 19-456) and TYR point mutants V377A, S380P and E345G.
2.2 Structural basis for efficient depigmenting agent design
Depigmenting agents are highly demanded in the cosmetic and pharmaceutical industry (Boissy et al., 2005; Chang, 2012; Kim et al., 2012). In the last decades, most of the tyrosinase inhibitors were developed using mushroom tyrosinase as a model enzyme (Chang, 2009; Ito and Wakamatsu, 2015; Xing et al., 2016; Yoshimori et al., 2014), and more recently, by computational docking on tyrosinase models obtained from crystal structures of bacterial and mushroom tyrosinases (Ai et al., 2014). However, due to the significant differences between mammalian tyrosinases and their lower organism counterparts, the resulting depigmenting agents may be unspecific for the human enzyme. Moreover, due to the intricate catalytic mechanism of tyrosinases, many phenolic compounds were misidentified as tyrosinase inhibitors, while they are actually alternative substrates and might thus generate highly reactive quinone molecules that exert cytotoxicity when used. Those compounds include hydroquinone (García-molina et al., 2014) and rhododendrol (Ito et al., 2014) among others, which were commonly used for cosmetic applications, but were later on banned because of their side effects (Medonald et al., 2001; Nishigori et al., 2015; O’Donoghue, 2006).

The crystal structure of human TYRP1 and the TYR structure model presented in this thesis provide a bona-fide platform for structure-based design of specific depigmenting agents. Furthermore, the ligand bound structures of TYRP1 and TYRP1-3M reveal detailed information about the binding mode of the ligands in the substrate binding pocket, which can be highly relevant for designing alternative compounds. In particular, these structures have enabled to identify the key residues that specifically interact with the inhibitors, namely Tyr362, Arg374, His381, Ser394, and Thr391. Besides, in the case of chelator-like inhibitors such as tropolone, the two zinc ions are the essential binding partners, in addition to the aromatic ring of His381 and the hydroxyl group of Ser394. Compared with TYRP1, the active site of TYR (as mimicked in TYRP1-3M) is somewhat more hydrophobic, although it also contains the conserved His381 and Ser394 essential elements, indicating that indeed the two proteins can bind common inhibitors.

Overall, although several mechanisms have been proposed to account for the differences between the catalytic activities of tyrosinases, the tyrosinase regulatory mechanism in the melanogenesis pathway is still poorly understood (D’Mello et al., 2016). Our results provide now for the first time structural insights into the catalytic features and the substrate/inhibitor binding mode of mammalian tyrosinases, which can contribute to decipher the atomic basis of melanogenesis regulatory mechanisms and related diseases, and pave the way for rational specific compound design.